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Genetic Marking of *Lactococcus lactis* Shows Its Survival in the Human Gastrointestinal Tract

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A human feeding study was performed with *Lactococcus lactis* TC165.5, which is genetically marked by insertion of the sucrose-nisin conjugative transposon Tn*5276* and chromosomal resistance to rifampin and streptomycin. The fate of strain TC165.5 and its nucleic acids was monitored by conventional plating methods and by molecular detection techniques based on specific PCR amplification of the nisin (*nisA*) gene from DNA extracted from human feces. A method was developed for the efficient extraction of microbial DNA from human feces. The results show that a fraction of viable cells of *L. lactis* TC165.5 survived passage through the human gastrointestinal tract. Only cells that passed within 3 days of ingestion could be recovered from the feces of the volunteers, and they accounted for approximately 1% of the total number of cells consumed. The presence of *nisA* in DNA extracted from feces could be detected up to 4 days, when viable cells were no longer present.

The use of lactic acid bacteria in the production of fermented foods has a long history (12). The application of these starter bacteria is aimed mainly at the production of foods with longer shelf life and better organoleptic properties. Much research effort has been focused on optimizing the performance of lactic acid bacteria during product formation or on broadening their applications, such as their use as probiotics (15) or as live vaccines (5, 14, 24).

The most commonly used starter bacteria include strains of *Lactococcus lactis*, which are used in the manufacturing of many cheeses and other fermented dairy products. In recent years, the genetics of mesophilic lactic acid bacteria have been greatly advanced and several tools have been developed for homologous and heterologous gene expression (5). This has resulted in the availability of genetically modified strains with prospects for applications in dairy product manufacturing and as oral vaccines (14, 24). For the application of genetically modified lactococci both as starter cultures and in health improvement, as a probiotic or a live vaccine, it is important to determine whether these bacteria survive in the gastrointestinal tract after consumption by humans.

The presence of *Lactococcus* spp. in the flora of the human gastrointestinal tract has been demonstrated in a few studies, but the discrimination between *Lactococcus* spp. and *Enterococcus* species is not always possible with classical identification methods (4). Stable populations of *L. lactis* can be established in monoassociated gnotobiotic mice, indicating that lactococcal strains can be maintained in an intestinal environment, although their optimum growth temperature is 30°C (8, 21). To obtain more information about the survival and the stability of lactococci in the human gastrointestinal tract, a study was performed with a genetically marked *L. lactis* strain in human feeding trials, during which the survival and persistence of the chromosomal DNA was monitored by conventional plating methods and molecular detection techniques (21).

DNA extraction from human feces. A sensitive detection method for *L. lactis* in human feces, based on DNA extraction and specific PCR amplification, was developed. Several methods have been described for the extraction of DNA from human fecal material (20, 22). We have developed a protocol based on the isolation of the microbial fraction, followed by cell lysis and chloroform-phenol extraction. The freshly collected fecal sample was stored at 4°C (up to 24 h), and 5 g was resuspended in 100 ml of a 300 mM sucrose solution. A 2-ml portion of this suspension was centrifuged at 2,750 × *g* for 10 min. The supernatant was removed, and the pellet was resuspended in 2 ml of sucrose solution. After centrifugation for 1 min at 750 × *g*, the supernatant, containing over 80% of the microbial fraction, was transferred to another tube. The efficiency of isolation of the microbial fraction was estimated by aerobic total plate counts of the suspension of freshly collected fecal sample on Columbia blood agar (Difco) plus 5% (vol/vol) sterile sheep blood and of the supernatant which contains the microbial fraction. The microbial fraction was collected by centrifugation at 2,750 × *g* for 10 min, and the cell pellet was resuspended in 1 ml of THMS (30 mM Tris · HCl [pH 8.0], 3 mM $MgCl_2$, 25% [wt/vol] sucrose) containing 2 mg of lysozyme per ml. Protoplasts were formed at 37°C for 1 h, after which the cells were lysed by adding 1 ml of TES (50 mM Tris · HCl [pH 8.0], 5 mM EDTA, 50 mM NaCl) containing 1% sodium dodecyl sulfate. DNA was obtained by chloroform-phenol extraction and ethanol precipitation (19) and further purified with genomic tips by following the manufacturer's protocol (QIAGEN, Chatsworth, Calif.). This preparation could be used in PCR without interference by inhibitory substances that are reported to be coextracted from feces (1).

Specific detection of the indicator strain by PCR amplification. *L. lactis* TC165.5 (18) was used as the indicator strain for consumption studies. This strain is a transconjugant of *L. lactis* MG1614 harboring a single copy of the sucrose-nisin transposon Tn*5276* (18). Strain MG1614 is a spontaneous mutant of the plasmid-free strain MG1363 (6) and shows resistance against rifampin and streptomycin. The presence of transposon Tn*5276* in strain TC165.5 allowed its specific enumeration on sucrose indicator plates consisting of Eliker agar (3) with 0.05% bromocresol purple and 0.5% sucrose and supplemented with rifampin (50 μg/ml) and streptomycin (100 μg/ml). MG1363 is a derivative of NCDO 712 and contains a 16S

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TABLE 1. Primers and probes used in this study

Primer or probe[a]	Target	Base positions	Sequence	Reference
Primers				
P1(S)	16S rRNA	41 to 60[b]	GCGGCGTGCCTAATACATGC	9
P2(A)	16S rRNA	686 to 705[b]	ATCTACGCATTTCACCGCTA	9
Pnis1(A)	Nisin	−99 to −78	CGCGAGCATAATAAACGGCT	12
Pnis2(S)	Nisin	201 to 220	GGATAGTATCCATGTCTGAAC	12
Probes				
Pgen(A)	16S rRNA	338 to 358[b]	CTGCTGCCTCCCGTAGGAGT	9
Pnis(S)	Nisin	1 to 20	ATGGGTTGTAATATGAAAAC	This study
PLc(A)	16S rRNA	V1 region	TTCAAATTGGTGCAAGCACC	12

[a] S, sense sequence; A, antisense sequence.
[b] *Escherichia coli* numbering is used.

rRNA sequence specific for *L. lactis* subsp. *cremoris* (7). Since the genotypically *L. lactis* subsp. *cremoris* indicator strain contains the structural nisin (*nisA*) gene, it could very well be distinguished from natural *L. lactis* subsp. *lactis* strains carrying the nisin operon (2).

To determine the sensitivity and specificity of the molecular detection method, different concentrations of *L. lactis* TC165.5 were added to fecal suspensions. The extracted DNA was used in a PCR amplification of the *nisA* gene with specific primers Pnis1 and Pnis2 (Table 1) (13). The products were blotted on a nylon membrane (GeneScreen plus; Dupont, Boston, Mass.) and hybridized with an internal specific probe Pnis (Table 1). The results (Fig. 1) show that it is possible to detect 10 cells per ml of resuspended feces (approximately 1,000 cells per g of feces). All extracted DNA was checked for the presence of PCR-inhibiting components by performing control amplifications with primers P1 and P2 based on conservative regions of the 16S rRNA, which, after blotting, were hybridized with the general 16S rRNA probe Pgen (Table 1) (9).

The use of specific PCR amplification in combination with an efficient DNA extraction of DNA from human fecal material is especially suitable for the specific and sensitive detection of bacteria which are present in relatively small numbers in human feces and are difficult to enumerate by conventional plating techniques, e.g., for the study of probiotic bacteria and the microbial ecology of the gastrointestinal tract.

Survival of lactococcal cells in the gastrointestinal tract. A double-blind human feeding trial was performed with six volunteers to establish whether lactococci survive the passage of the gastrointestinal tract (Fig. 2, experiment 1). This investigation was approved by the Medical Ethical Committee of Wageningen Agriculture University. Written informed consent was obtained from each subject. The six volunteers were divided into two groups; one group consumed a nonfermented product (four-fifths sterile milk and one-fifth orange juice [pH 5.2]) containing 10^8 cells of *L. lactis* TC165.5 per ml, and the other group received the same product but without added bacteria. The products were consumed twice a day for 4 days (first period). One week after the final consumption in the first period, a second consumption period started in which the type of product consumed by the two groups was reversed. Samples of feces and saliva were taken, as indicated in Fig. 2. Dilutions of the samples were plated on the sucrose indicator plates, on which *L. lactis* TC165.5 produces yellow colonies. The authenticity of the colonies appearing on the plates was verified by using the *nisA* and *L. lactis* subsp. *cremoris*-specific PCR primers (Table 1) by amplifying from DNA isolated from the colonies. All yellow colonies appeared to be *L. lactis* TC165.5. In some cases, white colonies with a dissimilar morphotype were detected on the plates; on microscopic analysis, these appeared to be yeasts. DNA was also extracted from the fecal samples and analyzed by specific PCR amplification for the presence of the *nisA* gene. Nonstimulated saliva samples, collected in sterile glass tubes 16 h after the fourth consumption of each period, were applied directly to the sucrose indicator plates. The indicator strain could not be recovered, showing that the strain does not persist in the oral cavity.

It is evident that *L. lactis* TC165.5 survived passage of the gastrointestinal tract in all volunteers (Table 2). A selection of

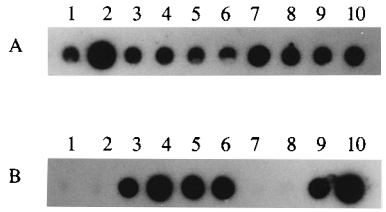

FIG. 1. Determination of the detection limit of *L. lactis* TC165.5 by PCR amplification. DNA was extracted from fecal suspensions (0.05 g [wet wt]/ml of 300 mM sucrose solution) to which different numbers of TC165.5 cells were added. This DNA was used in PCR amplification of the first half of the 16S rRNA with probes P1 and P2 (A) and of the *nisA* gene with probes Pnis1 and Pnis2 (B). Blots of the PCR products were hybridized with a general 16S rRNA DNA probe, Pgen (A), and a *nisA*-specific DNA probe, Pnis (B) (see Table 1 for a list of primers and probes). The following DNA solutions were used in the PCR amplifications: lanes 1 and 2, DNA isolated from fecal material with no TC165.5 added; lanes 3 to 8, 10^6, 10^5, 10^4, 10^3, 10^2, and 10 cells, respectively, of TC165.5 added per 5 g of fecal material; lane 9, purified DNA from TC165.5; lane 10, DNA isolated from fecal material to which purified TC165.5 DNA was added.

Outline experiment 1

day	1	2	3	4	5	12	14	15	16	17	18
group 1	C	C	CF_1	C	SF_2	F_3	C	C	CF_4	C	SF_5
(n=3)	Indicator strain						Placebo				
group 2	C	C	CF_1	C	SF_2	F_3	C	C	CF_4	C	SF_5
(n=3)	Placebo						Indicator strain				

S: saliva sample, F: fecal sample C: consumption of 2 X 100 ml product

Outline experiment 2

day	1	2	3	4	5	6	7	8	10	11
	FC	C	C	CF	F	F	F	F	F	F

F: fecal sample C: consumption of 100 ml product

FIG. 2. Outline of the two human feeding trials (experiments 1 and 2).

TABLE 2. Enumeration of *L. lactis* TC165.5 from the first human feeding experiment

Order of indicator and placebo administration	Person	*L. lactis* TC165.5 counts (log CFU/g of feces)[a]				
		Week 1		Week 2,	Week 3	
		F_1	F_2	F_3	F_4	F_5
Placebo/indicator	1	<2[b]	<2	<2	5.38	5.66
Indicator/placebo	2	3.45	6.53	<2	<2	<2
Indicator/placebo	3	5.08	4.62	<2	<2	<2
Placebo/indicator	4	<2	<2	<2	4.30	3.41
Indicator/placebo	5	3.98	3.83	<2	<2	<2
Placebo/indicator	6	<2	<2	<2	2.25	4.50

[a] F_1 to F_5, fecal samples in experiment 1 (Fig. 2).
[b] <2, below detection limit.

isolates were analyzed by specific PCR; they all contained the *nisA* gene and were identified as *L. lactis* subsp. *cremoris* (data not shown). The number of cells (approximately 10^4/g of feces) was such that on general specific media, they would have been overgrown by *Enterococcus faecalis* and other streptococci, which are generally present at concentrations of 10^8 cells per g of feces (4).

The results of the molecular detection experiment (Fig. 3) show that the *nisA* gene was present only in samples that contained viable cells of the indicator strain, although even samples containing less than 10^3 cells per g of feces (established detection limit, Fig. 1) yielded positive signals.

Quantification of the survival rate. To quantify the survival of *L. lactis* TC165.5 in the gastrointestinal tract, a second human feeding trial was performed (Fig. 2, experiment 2). For 4 days, four volunteers consumed (per day) 100 ml of a product similar to that in the previous experiment, containing 10^9 *L. lactis* cells per ml. In addition to lactococci, *Bacillus stearothermophilus* spores were added to the product as a microbial passage marker (10^5 spores [Merck, Darmstadt, Germany] per ml of product). The spores do not grow at 37°C and do not germinate in the gastrointestinal tract. Spores in the fecal samples were enumerated on plate count agar (Difco, Detroit, Mich.) incubated at 65°C (16). During a period of 9 days, all fecal material was collected and analyzed by selective plate counting and PCR amplification of the *nisA* gene.

In the fecal material collected shortly after consumption, about 0.1 to 2% of *L. lactis* TC165.5 cells survived passage through the intestinal tract (Fig. 4). After 2 days, the number of cells that had survived decreased rapidly, and after 3 days, no viable cells could be detected (detection limit, 10^2 cells per g of feces in the plate counts). Excretion of spores, however remained at a constant level for 4 days and then dropped gradually to below the detection limit on day 8 (Fig. 4). This result is in agreement with the passage kinetics of *B. stearothermophilus* spores observed by other investigators, and comparable passage kinetics have been found for *Bifidobacterium* spp., indicating that they can survive very well in the gastrointestinal environment (16, 17). The lactococcal counts in feces (Fig. 4) suggest that during the 4 days when the passage of spores is at a constant level, the numbers of lactococci decrease according to a first-order kinetics, indicating that the viability of the consumed lactococci declines consistent with the response of a homogeneous population to stress (10).

The decline of living lactococcal cells is also reflected in the gradual disappearance of DNA coding for the *nisA* gene in the fecal material after 3 to 4 days. Similar to the first experiment, the PCR detection method appeared to be more sensitive than the plating method, since some samples in which no viable cells could be detected showed a positive signal in the PCR amplification. This suggests the presence of nonculturable cells of TC165.5 or of naked DNA derived from lysed cells. Such DNA might be protected against nuclease activity by binding to particles, as has been demonstrated in soil and other environments (11).

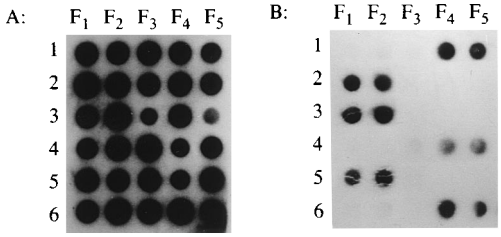

FIG. 3. Detection of the indicator strain *L. lactis* TC165.5 in human feces obtained in experiment 1. (A) Results of the PCR amplification of the first part of the 16S rRNA (with probes P1 and P2) and the hybridization with the general 16S rRNA probe Pgen (control for the absence of PCR-inhibiting components in the DNA isolation). (B) Results of the PCR amplification of the *nisA* gene (with probes Pnis1 and Pnis2) and the hybridization with the *nisA*-specific DNA probe Pnis are shown. Rows 1 to 6, volunteers; columns F_1 to F_5, fecal samples (Table 2).

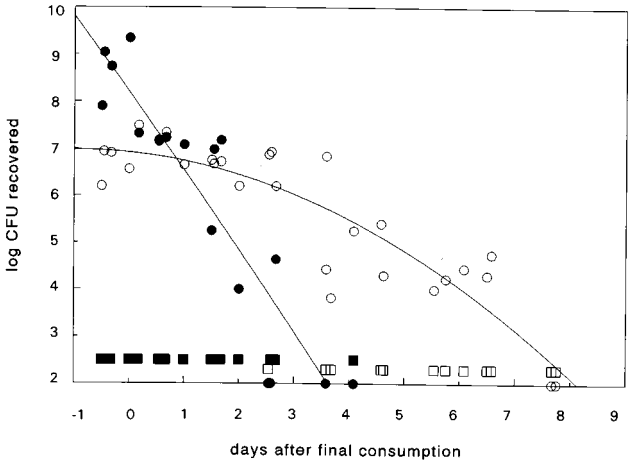

FIG. 4. Recovery of *L. lactis* TC165.5 and *B. stearothermophilus* spores in the feces of four volunteers. Viable counts of *L. lactis* TC165.5 and *B. stearothermophilus* recovered from fecal samples obtained from four volunteers during experiment 2 (outline in Fig. 2) are shown. In addition, the persistence of the *nisA* gene was determined in the same samples by specific PCR. 0 represents the time of final consumption. Symbols: ●, *L. lactis* TC 165.5; ○, *B. stearothermophilus* spores; ■, positive PCR signal for *nisA*; □, negative PCR signal for *nisA*. When general PCR primers were used, all DNA extractions were shown to be free from PCR-inhibiting factors.

This study shows that a substantial proportion of *L. lactis* cells, consumed in a dairy product, survive the passage of the gastrointestinal tract, provided that they do pass within 3 days after consumption. This is in agreement with in vitro work (23) showing that lactococci had a relatively high resistance to conjugated bile salts, comparable to that of intestinal bacteria. Both the fact that only up to 2% of the total amount of consumed bacteria are recovered and the fact that their numbers in the feces decrease more rapidly than those of the microbial passage marker, however, indicate that the viability of the lactococci is negatively influenced by the gastrointestinal environment. The question whether they are metabolically active in the gastrointestinal tract still remains to be answered. The partial survival of lactococci gives a positive prospective for the use of *Lactococcus* strains in the development of oral vaccines (12). On the other hand, the survival of lactococcal cells in the gastrointestinal tract may have consequences for the evaluation of genetically modified strains, notably those containing antibiotic resistance genes, used in the production of foods. It has been shown that gene transfer between lactococcal cells and other microorganisms, especially via conjugation, is possible in the gastrointestinal environment (7). The experimental outline in this study has been shown to be suitable for determining the fate of microorganisms and their nucleic acids during the passage of the gastrointestinal tract and can be used in future research on probiotics and on the risk assessment of the use of genetically modified microorganisms in food.

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